1   **Interferon signaling suppresses the unfolded protein response and induces cell death in**

2   **hepatocytes accumulating hepatitis B surface antigen**

3

4   Short title: IFNs induce cell death in hepatocytes accumulating hepatitis B surface antigen

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Abstract

48 Virus infection, such as hepatitis B virus (HBV), often causes endoplasmic reticulum (ER)

49 stress. The unfolded protein response (UPR) is counteractive machinery to ER stress, and the

50 failure of UPR to cope with ER stress results in cell death. Mechanisms that regulate the

51 balance between ER stress and UPR in HBV infection is poorly understood. Type 1 and type

52 2 interferons have been implicated in hepatic flares during chronic HBV infection. Here, we

53 examined the interplay between ER stress, UPR, and IFNs using transgenic mice that express

54 hepatitis B surface antigen (HBsAg) (HBs-Tg mice) and humanized-liver chimeric mice

55 infected with HBV. IFNα causes severe and moderate liver injury in HBs-Tg mice and HBV

56 infected chimeric mice, respectively. The degree of liver injury is directly correlated with

57 HBsAg levels in the liver, and reduction of HBsAg in the transgenic mice alleviates IFNα

58 mediated liver injury. Analyses of total gene expression and UPR biomarkers' protein

59 expression in the liver revealed that UPR is induced in HBs-Tg mice and HBV infected

60 chimeric mice, indicating that HBsAg accumulation causes ER stress. Notably, IFNα

61 administration transiently suppressed UPR biomarkers before liver injury without affecting

62 intrahepatic HBsAg levels. Furthermore, UPR upregulation by glucose-regulated protein 78

63 (GRP78) suppression or low dose tunicamycin alleviated IFNα mediated liver injury. These

64 results suggest that IFNα induces ER stress-associated cell death by reducing UPR. IFNγ

65 uses the same mechanism to exert cytotoxicity to HBsAg accumulating hepatocytes.

66 Collectively, our data reveal a previously unknown mechanism by which IFNs selectively

67 induce cell death in virus-infected cells. This study also identifies UPR as a potential target

68 for regulating ER stress-associated cell death.

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71 **Author summary**

72 Hepatitis B virus (HBV) causes acute and chronic infections that kill over 600,000 people

73 every year from severe hepatitis, liver cirrhosis, and cancer. Mechanisms of chronic liver

74 injury remain largely unknown. Both type 1 and type 2 interferons (IFNs) have been

75 implicated in hepatic flares during chronic HBV infection, although HBV per se is a poor

76 IFN inducer. In addition, while IFNα, a type 1 IFN, used to be the first-line treatment for

77 chronic hepatitis B (CHB) patients, adverse side effects, including hepatic flares, severely

78 limit their therapeutic effectiveness. These clinical observations suggest a pathogenic role of

79 IFNs in HBV infection. Here, we demonstrate that IFN-1s cause severe and moderate

80 hepatitis in transgenic mice expressing hepatitis B surface antigen (HBs-Tg mice) and human

81 hepatocyte chimeric mice infected with HBV, respectively. HBsAg accumulation appears to

82 cause ER stress because a counteractive response to ER stress, namely, unfolded protein

83 response (UPR), was induced in both HBs-Tg mice and HBV infected chimeric mice. Our

84 results indicate that IFN-1s suppress UPR before causing liver injury. UPR was also

85 suppressed by IFNγ. Induction of UPR in HBs-Tg mice before treatment with IFNα and IFNγ

86 significantly alleviated liver injury. We suggest that IFNs exert cytotoxicity to ER stress

87 accumulating cells by suppressing UPR.

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89 **Keywords**

90 Interferons, ER stress, unfolded protein response, cell death, hepatitis B virus, surface

91 antigen, liver injury

Introduction

Interferons (IFNs) play a critical role in host defense against pathogens, particularly viruses, by activating the expression of hundreds of genes that exert antiviral activity [1]. IFNs also cause immunopathology during viral infections [2,3]. The basis behind the IFN-mediated immunopathology has yet to be fully elucidated but appears to include phosphorylation of eukaryotic initiation factor-2α (eIF-2α), activation of RNase L, and induction of nitric oxide synthase (iNOS) [1,2]. Little is known about whether and how infected cells are preferentially sensitized to IFN-mediated cell death.

The endoplasmic reticulum (ER) is responsible for much of a cell's protein synthesis and folding. An imbalance between the protein-folding load and the capacity of the ER causes unfolded or misfolded proteins to accumulate in the ER lumen, resulting in ER stress [4,5]. To cope with ER stress, a protective mechanism called the unfolded protein response (UPR) is activated to reduce protein synthesis and/or enhance degradation, folding, and secretion of the offending proteins [4,5]. UPR has been linked to various pathological states, including malignancies, neurodegenerative, storage, metabolic, and infectious diseases [6–11]. While unmitigated ER stress leads to cell death [12], it is unclear whether the concomitant UPR plays a pro- or anti-cell survival role in such cases. Maladaptation of UPR as the underlying pathogenic mechanism has been poorly studied, particularly in highly secretory organs such as the liver and pancreas that are prone to ER stress [13]. Although infections with several viruses have been reported to induce ER stress [14–19], the interplay between ER stress, UPR, and IFN signaling has not been adequately interrogated.

Hepatitis B virus (HBV) causes acute and chronic liver disease. Patients with chronic hepatitis B (CHB) often experience hepatic flares in association with hepatitis B surface antigen (HBsAg) accumulation [20,21]. Factors that cause hepatic flares in CHB patients are

116   incompletely understood. IFNα, a type-1 IFN (IFN-1), is widely used as the first-line drug

117   for the treatment of chronic hepatitis B (CHB) [22]. High serum IFNα levels have been

118   reported in some CHB patients experiencing spontaneous hepatic flares [23] or antiviral

119   therapy withdrawal-associated hepatic flares [24]. However, the molecular mechanisms

120   behind the IFN-1 related liver injury largely remain obscure. In addition, CXCL10, an IFNγ

121   inducible chemokine, has been associated with disease progression [25] as well as HBsAg

122   clearance in CHB patients [26]. In this study, we examined the role of IFNs in liver injury

123   associated with ER stress using transgenic mice that express HBsAg in the liver (HBs-Tg

124   mice) and HBV-infected humanized-liver chimeric mice. Our data suggest that IFNs

125   selectively causes cell death in hepatocytes under ER stress by perturbing UPR.

Results

Type-1 IFNs induce liver injury in association with HBsAg-retention.

To investigate the role of IFN-1 signaling in liver injury associated with intrahepatic HBsAg accumulation, we used HBs-Tg mice (lineage 107-5D) that produce HBsAg [27]. Groups of 3-4 HBs-Tg mice or their non-transgenic littermates (WT) were intravenously injected with the IFN-1 inducer poly I:C or saline. Liver injury was monitored by measuring serum ALT activity (sALT) on days 1, 3, and 7 after treatment. As shown in Fig 1A, sALT was markedly elevated in the HBs-Tg mice, peaking on day 1 after poly I:C treatment, but not in the WT mice. We also examined whether poly I:C treatment could induce sALT elevation in HBV-replication-competent transgenic (HBV-Tg) mice (Lineage 1.3.32) [28]. As shown in Fig 1B, HBV-Tg mice readily secrete HBsAg, retaining approximately 100-fold less HBsAg in the liver compared with the HBs-Tg mice. Interestingly, no ALT elevation occurred in the HBV-Tg mice after poly I:C treatment (Fig 1C). Taken together, these results suggest that poly I:C-induced liver injury is associated with marked intrahepatic HBsAg accumulation.

Fig 1. Poly I:C induces liver injury in association with HBsAg-retention. (A) Serum ALT levels measured on days 0, 1, 3, and 7 after PBS (white bars) or poly I:C (10 μg) (red bars) treatment in HBs-Tg mice (left graph) and WT mice (right graph). (B) Comparison of HBsAg expression levels between HBs-Tg (Lineage 107-5D: white bars) and HBV-Tg mice (Lineage 1.3.32: grey bars) in the serum (left graph) and the liver (right graph). (C) Serum ALT levels in HBs-Tg mice and HBV-Tg mice 24 hours after intravenous injection of PBS or poly I:C. Mean values +/- s.d of pooled data from 3 independent experiments are shown.

149   To examine the role of IFN-1 signaling in the poly I:C induced liver injury in HBs-Tg

150   mice, groups of HBs-Tg mice (n=4) were treated with anti-IFNα/β receptor 1 (IFNαβR1)

151   antibody that blocks IFN-1 signaling or a control antibody and then injected with poly I:C 24

152   hours later. The impact of IFN-1 signaling blockade was evaluated by monitoring sALT

153   activity. As shown in Fig 2A, anti-IFNαβR1 antibody but not the control antibody treatment

154   prevented sALT elevation, indicating that the liver injury induced after poly I:C treatment in

155   HBs-Tg mice was dependent on IFN-1 signaling. To confirm that IFN-1 induces liver injury,

156   groups of HBs-Tg mice (n=3) and control mice (n=3) were intravenously injected with 5

157   million units (MU)/kg of recombinant mouse IFNα, and sALT was monitored on days 1, 3,

158   and 7. As expected, IFNα induced sALT elevation in the HBs-Tg mice, peaking on day 1 and

159   receding towards baseline on day 3. No sALT elevation occurred in the normal mice (Fig

160   2B). Interestingly, liver immunohistochemical analysis showed almost no inflammatory cell

161   infiltration at 24 hours despite severe sALT elevation, suggesting a sterile nature of IFNα

162   mediated cell-death (Fig 2C). When apoptosis was examined by TUNEL staining at 16 hours

163   after IFNα, a relatively small fraction of TUNEL positive hepatocytes were found scattered

164   throughout the parenchyma (Fig 2D).

165   **Fig 2. IFN-1s trigger HBsAg-associated liver injury in the absence of inflammatory cell**

166   **infiltration.** (A) Isotype control antibodies (250 μg: red bars) or anti-IFNαβR1 (250 μg:

167   black bars) were intraperitoneally injected into HBs-Tg mice (n=3-4) 24 hours before poly

168   I:C treatment. ALT levels on days 0, 1, 3, and 7 after poly I:C treatment are shown. (B)

169   Serum ALT values in HBs-Tg (left graph) and WT mice (right graph) on days 0, 1, 3, and 7

170   after intravenous injection of PBS (white bars) or IFNα (5 million units (MU)/kg: blue bars).

171   (C) Histological characteristics after IFNα injection in HBs-Tg mice. The panels show

172   representative Hematoxylin and Eosin staining at 24 hours after IFNα. (D) TUNEL staining

173   at 16 hours after IFNα treatment. The arrows show representative apoptotic cells in HBs-Tg

174   mice.

175

176   To examine the impact of HBsAg reduction on IFN-mediated liver injury, HBs-Tg

177   mice were injected by siRNA targeting HBV (siHBV) that were shown to reduce HBV

178   mRNA in HBV-infected human hepatocyte chimeric mice [29,30] or control siRNA, and

179   then intrahepatic HBsAg and HBV mRNA levels were determined 4 days later (S1A Fig). As

180   shown in S1B Fig, siHBV had no discernable effect on HBsAg, although it strongly

181   suppressed HBV mRNA expression (S1C Fig), indicating the stable nature of intracellular

182   HBsAg. To induce hepatocyte turnover, we adoptively transferred HBsAg-specific T-cells

183   that were shown to induce severe hepatitis in HBs-Tg mice [31] to groups of HBs-Tg mice

184   (n=4) seven days before injecting siHBV or siControl. As shown in Fig 3B, HBsAg was

185   significantly reduced on day 7 after siHBV treatment when hepatocyte turnover was

186   previously induced. These mice were then injected intravenously with recombinant mouse

187   IFNα on day 7 after siHBV treatment. A day after IFNα injection (day 8), serum ALT levels

188   were significantly reduced in siHBV treated mice compared with their siControl treated

189   littermates (sALT 425±103 vs. 4227±2533, p=0.027) (Fig 3C). These results indicate that

190   IFNα mediated liver injury can be prevented by the reduction of intrahepatic HBsAg levels.

191   **Fig 3. Reduction of intrahepatic HBsAg alleviates IFN mediated liver injury.** (A)

192   Experimental design. HBs-Tg mice were adoptively transferred with HBsAg-specific effector

193   CD8 T cells on day 7 day before siRNA treatment. On day 7 after siRNA treatment, mice

194   were intravenously injected with IFNα (5 MU/kg) and, on day 8, monitored for sALT levels.

195   (B) Suppression of intrahepatic HBsAg expression before IFNα treatment (day 7 after siRNA

196   treatment). (C) Serum ALT levels before (day 7) and after (day 8) IFNα treatment.

197

IFN-1 signaling perturbs UPR in association with liver injury in HBs-Tg mice.

199   To elucidate the molecular mechanism by which IFN-1 induces liver injury in HBs-

200   Tg mice, we serially profiled the liver transcriptomes of both HBs-Tg and control mice

201   treated with IFNα by microarray analyses. We especially searched for distinct genes whose

202   expression correlated with the IFNα mediated liver injury in HBs-Tg mice. To do so, snap-

203   frozen liver samples were obtained from groups of four HBs-Tg and control mice sacrificed

204   prior to (0 hour) and 2, 4, 8, and 24 hours after treatment with 5 MU/kg of IFNα or vehicle.

205   Liver injury progression was also monitored at these time points using sALT. As shown in

206   Fig 4A, a significant sALT increase was observed in HBs-Tg mice from 8 hours post-IFN

207   treatment and peaked at 24 hours (3758 ± 649 U/L). As expected, no sALT elevation

208   occurred in the WT mice. Cluster and enrichment analyses were performed on the

209   microarray data for the selected period prior and up to the onset of liver injury, i.e., 0, 2, 4,

210   and 8-hour time-points. As shown in Figs 4B and 4C, six clusters showing distinct gene

211   expression profiles were identified. Many ISGs could be found in TLR signaling and

212   chemokine signaling pathways of Cluster 6 (Fig 4C), which were induced similarly between

213   HBs-Tg and WT mice (Figs 4B and 4C). Cluster 3 genes were upregulated in HBs-Tg mice

214   before IFN treatment, and as ISGs were induced, they were downregulated in both HBs-Tg

215   and WT mice up to 4 hours (Fig 4B), suggesting a suppressive effect of ISGs on these genes.

216   Interestingly, while the expression of these genes rebounded in WT mice, they remained

217   downregulated in HBs-Tg mice at 8 hours after IFN treatment. Importantly, Cluster 3

218   includes genes related to protein processing in the endoplasmic reticulum (Fig 4C), raising

10

219   the possibility that IFNα induced liver injury in HBs-Tg mice by modulating intrahepatic

220   UPR. To test this, the correlation between liver injury, IFN-1 signaling, and UPR-related

221   molecule expression was further examined. Intrahepatic induction of interferon-stimulated

222   genes (ISGs), UPR biomarkers such as spliced X-box binding protein-1 (XBP1s)

223   phosphorylated- PKR-like ER kinase (phos-PERK) and C/EBP homologous protein (CHOP)

224   (Fig 4D) were assessed by western blot. As shown in S2A Fig, non-treated HBs-Tg mice

225   showed higher baseline expression of XBP-1s, phos-PERK, and CHOP in association with

226   moderate liver injury (sALT 100-300 U/L) than non-treated WT mice (S2B Fig), suggesting

227   that HBsAg accumulation triggered UPR activation in association with mild spontaneous

228   hepatitis. Interestingly, as shown in Fig 4E, the expression of XBP1-s and phos-PERK were

229   transiently suppressed during ISG induction, in concordance with the mRNA levels

230   represented in Cluster 3 of the microarray data (Figs 4B and 4C). As XBP1s and phos-PERK

231   returned towards baseline levels, CHOP began to increase markedly in HBs-Tg mice from 8

232   hours and peaked at 24 hours in direct correlation with sALT. An ER chaperone molecule,

233   glucose-regulated protein 78 (GRP78) was also upregulated in HBs-Tg mice at 24hours (Fig

234   4E), in association with peak liver injury (Fig 4A). The aforementioned changes were not

235   observed in WT mice, although XBP1 expression was modestly reduced immediately after

236   IFNα treatment (Fig 4E). These data suggest that IFN-signaling induces liver injury in HBs-

237   Tg mice in association with UPR modulation.

238   **Fig 4. IFN-1 signaling perturbs UPR in association with liver injury in HBs-Tg mice.**

239   (A) Liver injury progression after IFNα treatment. The graph shows sALT levels in WT

240   mice (white bars) and HBs-Tg mice (black bars) after IFNα treatment (5MU/kg). Mean

241   values +/- s.d of pooled data from 3 independent experiments are shown. (B) Differentially

242   expressed genes (DEGs) in the microarray data of HBs-Tg and WT mice after IFNα

243   treatment. Each of the 6 graphs shows a distinct cluster identified using microarray time-

244   course-specific maSigPro [32] software. (C) List of gene pathways selectively enriched in the

245   6 clusters. (D) A schematic diagram showing simplified unfolded response pathways. (E)

246   Representative immunoblots showing UPR-biomarker protein expression in HBs-Tg mice

247   (left panel) and WT mice (right panel) at specified time points after IFNα treatment.

248

249   **IFNα induces liver injury in HBV infected human chimeric mice in association with**

250   **XBP1 suppression.**

251   To examine the effect of IFNα treatment on UPR in HBV infected human liver, we

252   used human liver chimeric mice, i.e., uPA-SCID mice whose livers had been repopulated

253   with human hepatocytes as previously described [33]. We first compared circulating and

254   intracellular HBsAg levels between HBV-Tg mice, HBs-Tg mice, and chimeric mice infected

255   with HBV for 6 weeks. As shown in S3 Fig, HBV infected chimeric mice secreted more

256   HBsAg than HBV-Tg mice (>10-fold difference) and HBs-Tg mice (>100-fold difference)

257   (S3A Fig) but still retained more HBsAg in the liver than 1.3.32 HBV-Tg mice (S3B Fig).

258   Next, groups of nine chimeric mice were infected with HBV (genotype C isolate,

259   C2_JPNAT, from a chronic HBV patient[33]), and after the establishment of persistent

260   infection (6-8 weeks after HBV inoculation), the mice were intravenously administered with

261   25 ng/g of pegylated-human IFNα (PEG-hIFNα), and then sacrificed after 8 and 24 hours

262   (n=3 per group) (Fig 5A) to examine the correlation between liver injury (sALT) (Fig 5B),

263   IFN-1 signaling, and UPR-related molecules measured by quantitative-PCR (Fig 5C) and

264   western blot (Fig 5D). Non-infected, non-treated control chimeric mice (n=2) were also

265   sacrificed and included in the analyses. Before PEG-hIFNα treatment, HBV infected

266   chimeric mice showed slightly higher sALT activity than untreated mice (Fig 5B, mean 268

267   vs. 130 U/L, p=0.001) in association with modest intrahepatic upregulation of UPR

268   molecules including XBP1s and PERK (Fig 5C). Importantly, sALT levels increased in HBV

269   infected chimeric mice at 8 (724 ± 159 U/L) and 24 hours (616 ± 26 U/L) after PEG-hIFNα

270   treatment (Fig 5B) in association with an increased level of ISG15 expression and reduced

271   levels of UPR-related molecules compared to non-treated HBV infected mice (Fig 5C and

272   5D). Intracellular HBsAg levels were mostly unchanged during the 24-hour period (Fig 5D),

273   suggesting that the suppression of UPR-related molecules, including XBP1s, was not due to a

274   reduction of HBV surface antigens. Collectively, these results indicate that bona fide HBV

275   infection could induce UPR in human hepatocytes, and IFN-1 signaling induces liver injury

276   in association with UPR suppression.

277   **Fig 5. IFNα induces liver injury in HBV infected human chimeric mice in association**

278   **with XBP1 suppression.** (A) Experimental design. HBV infected mice were injected with 25

279   ng/g pegylated IFNα-2a and then sacrificed at specified time points (n=3 per time point). (B)

280   Liver injury after IFN treatment in HBV infected chimeric mice. The graph shows serum

281   ALT levels at baseline (0 h), 8, and 24 hours after IFN treatment (black bars) compared with

282   non-infected non-treated chimeric mice (white bars). Data are shown as mean values +/- s.d.

283   (C) Messenger RNA expression levels of UPR biomarkers after IFN treatment. Levels of

284   UPR related molecule mRNA expression determined by quantitative PCR. (D) Association

285   between XBP, ISG15, and HBV surface antigen (HBsAg) after IFN treatment.

286   Representative immunoblots showing UPR biomarkers XBP1, ISG15, and HBsAg levels at

287   0, 8, and 24 hours after IFN treatment.

288

289   **IFNs exert direct cytotoxicity to HBsAg accumulating hepatocytes and downregulate**

290   **UPR.**

291   To dissect the mechanism of the IFNα-mediated liver injury, we established a

292   primary mouse hepatocyte (PMH) culture system amenable to knockdown experiments.

293   Primary hepatocytes from both HBs-Tg and WT mice were treated with IFNα or medium,

294   and then cytotoxicity and UPR-related protein expression were assessed 8, 24, and 72 hours

295   later (Fig 6A). The degree of cytotoxicity was analyzed by calculating the percentage of

296   specific cell death based on LDH activity in the supernatant as described in the Materials and

297   Methods. As shown in Fig 6B, cytotoxicity was clearly observed at 72 hours after IFNα

298   treatment in the HBs-Tg PMHs but not in the WT PMHs, indicating that IFNα is directly and

299   specifically cytotoxic to HBsAg accumulating hepatocytes. Interestingly, UPR markers such

300   as XBP1s, phosho-PERK, and CHOP expression were upregulated from 24 hours, and then

301   diminished at 72 hours coinciding with ISG induction and cytotoxicity. Furthermore, GRP78

302   was upregulated at 72 hours in association with cytotoxicity. No UPR modulation was

303   observed in the WT PMHs (Fig 6C). These in vitro data closely recapitulate our observations

304   in vivo despite the slower temporal dynamics of ISG induction in vitro and lower baseline

305   UPR in HBsAg PMHs. To determine whether the observed suppressive effect of IFNα on

306   UPR was a general occurrence or restricted to the HBs-Tg model, we tested the impact of

307   IFN-1 signaling on UPR that was chemically induced by thapsigargin in normal PMHs at 6

308   and 12 hours (Fig 6D). Interestingly, thapsigargin-induced Perk and CHOP but not XBP1s

309   expression were clearly suppressed by IFNα treatment at both time points (Fig 6E),

310   suggesting that UPR suppression by IFNα was in part a general phenomenon.

311   **Fig 6. IFNα exerts direct cytotoxicity to HBsAg accumulating hepatocytes and**

312   **downregulate UPR.** (a-c) Effects of IFN signaling and UPR modulation on HBsAg

313   accumulating hepatocytes in vitro. (A) Experimental design. (B) LDH levels in the

314   supernatant of PMH culture were measured at indicated time points after adding medium

14

315   (white bars) or IFNα (0.1MU/ml) (black bars). (C) The immunoblots of UPR related

316   molecules and ISG15 at specified time points after IFNα treatment. (D, E) IFNα suppresses

317   chemically induced UPR in vitro. (D) Experimental schema. (E) Representative

318   immunoblots show the effect of IFNα on UPR related-protein levels at 0, 6, and 12 hours

319   after treatment with thapsigargin.

320

321   We also tested the direct effect of IFNγ on hepatocytes accumulating HBsAg using

322   the previously described in-vitro culture system (S4A Fig), because IFNγ has been reported

323   to induce liver injury in mice that retain HBsAg in the liver [31,34]. As expected, IFNγ

324   (10,000 U/ml) directly and specifically induced cell death in HBs-Tg PMHs (S4B Fig).

325   Again, UPR was upregulated at 24 hours and then downregulated at 72 hours in association

326   with cytotoxicity and GRP78 upregulation (S4C Fig), similar to IFNα.

327   **GRP78 suppression reduces IFN-induced hepatocytotoxicity by upregulating UPR.**

328   To determine the role of UPR related molecules in IFNα-mediated

329   hepatocytotoxicity, CHOP, GRP78 and XBP1 were downregulated by transfecting HBs-Tg

330   PMHs with target-specific siRNA or a scrambled siRNA control for 24 hours before addition

331   of IFNα (Fig 7A). Cytotoxicity was assessed by measuring secreted LDH levels in culture

332   supernatants 72 hours after IFNα addition. Surprisingly, suppression of GRP78 but not

333   CHOP or XBP1 reduced LDH (Fig 7B) in association with upregulation of UPR related

334   molecules, including PERK and XBP1s (Fig 7C). To determine whether the reduction in

335   IFNα cytotoxicity by siGRP78 was due to the UPR upregulation, GRP78 and the key UPR

336   molecule XBP1 were co-suppressed prior to IFNα treatment. Interestingly, the reduction of

337   cytotoxicity by siGRP78 was lost when XBP1 was co-suppressed (Figs 7D and 7E),

338   suggesting that UPR is required for the reduction of cytotoxicity by GRP78 suppression.

339   These results suggest that robust UPR activation by GRP78 suppression rescues HBsAg

340   accumulating hepatocytes from the cytotoxic effect of IFNα.

341   **Fig 7. GRP78 suppression reduces IFNα-induced hepatocytotoxicity in association with**

342   **UPR upregulation.** (A) Experimental design. Small interfering RNA (siRNA) targeting

343   CHOP, GRP78, XBP1, or control scramble siRNA (siControl) (15 µM) were transfected to

344   primary mouse hepatocytes (PMHs) from HBs-Tg and WT mice before IFNα treatment. (B)

345   LDH levels in the supernatant of cultured HBs-Tg PMHs after IFNα treatment following

346   knockdown of each UPR-related molecule by specific siRNA. (C) Representative

347   immunoblots showing UPR-related protein levels in the absence/presence of IFNα after

348   indicated specific target downregulation by siRNA. (D) LDH levels in the supernatant of

349   cultured HBs-Tg PMHs after IFN treatment following control, GRP78, and GRP78/XBP1 co-

350   suppression by siRNA. (E) UPR-related protein levels in the absence/presence of IFNα in

351   siControl, siGRP78, and siGRP78/siXBP1 suppressed HBs-Tg PMHs. Mean values +/- s.d of

352   pooled data from 2 independent experiments are shown.

353

354   **UPR upregulation alleviates IFN-induced liver injury in HBs-Tg mice.**

355   To determine whether GRP78 suppression could alleviate liver injury in HBs-Tg

356   mice, we intravenously injected siRNA targeting GRP78 (siGRP78), or control siRNA, into

357   groups of 3-4 HBs-Tg mice 4 days before IFNα treatment (Fig 8A). As shown in Fig 8B,

358   siGRP78 treated mice exhibited 3-5-fold lower sALT levels after IFNα treatment compared

359   to control siRNA treated mice (mean 7110 ± 42 U/L vs. 1610 ± 794 U/L, p=0.003). These

16

360 data suggest that robust UPR induction prevents IFN-induced liver injury in HBs-Tg mice.

361 Therefore, the impact of UPR augmentation on IFN-mediated liver injury was tested using a

362 chemical UPR inducer, tunicamycin. Groups of HBs-Tg and WT mice (n=3) were

363 intraperitoneally injected with a low dose of tunicamycin (0.1 mg/kg) or saline. Significant

364 UPR upregulation could be seen 4 hours after tunicamycin treatment (Fig 8C), at which time

365 point IFNα (5 MU/kg) or 50 ng α-Galactocsylceramide (αGal; an IFNγ inducer) was

366 injected into these mice. The levels of sALT activity and intrahepatic UPR-related molecule

367 expression were measured 24 hours after IFNα or αGal treatment. As shown in Fig 8D,

368 sALT elevation was almost completely blocked in the tunicamycin pre-treated animals

369 compared to the vehicle-treated control group after IFNα (3665 ± 500 U/L vs. 207 ± 73 U/L,

370 p<0.001). Tunicamycin treatment also suppressed IFNγ-mediated liver injury in HBs-Tg

371 mice as tunicamycin treated mice showed up to a 6-fold reduction in sALT levels compared

372 to the controls after αGal injection (Fig 8E; 12020 ± 1100 U/L vs. 1940 ± 388 U/L, p<0.001).

373 These data indicate that UPR augmentation rescues HBsAg accumulating cells from the

374 cytolytic effect of type I and type II IFNs. The data also suggest that IFNα and IFNγ utilize a

375 similar molecular mechanism to induce cell death in HBsAg accumulating hepatocytes (Fig

376 8F).

377 **Fig 8. UPR upregulation alleviates IFN-induced liver injury in HBs-Tg mice.** (A)

378 Experimental design to test the effect of GRP78 suppression on IFN-induced liver injury. (B)

379 sALT levels at 24 hours after IFNα injection in siGRP78 treated HBs-Tg mice compared

380 with controls. (C) Experimental design to test the effect of tunicamycin administration

381 (0.1mg/kg) on IFN-induced liver injury. IFNα (5 MU/kg) or α-Galactocsylceramide (αGal)

382 (50ng/mouse) were intravenously injected to HBs-Tg mice at 4 hours after tunicamycin

383 administration. The immunoblots below show the intrahepatic level of UPR markers at

384   indicated time points. (D) sALT levels at 24 hours after IFNα injection in tunicamycin

385   (TUN) treated HBs-Tg mice compared with controls. (E) sALT levels at 24 hours after αGal

386   injection in tunicamycin and vehicle-treated HBs-Tg mice. Mean values +/- s.d of pooled

387   data from 3 independent experiments are shown. (F) A schema depicting our hypotheses on

388   the effect of IFN-1 signaling on UPR to induce ER stress-related cell death in HBsAg

389   accumulating hepatocytes.

390

391

Discussion

392   In this study, we examined whether and how IFNs induce liver injury in association

394   with HBsAg accumulation in the liver. IFNα directly and specifically induced cell death in

395   HBsAg accumulating hepatocytes in association with suppression of the pro-survival UPR.

396   IFNγ appears to use the same mechanism to cause cellular damage. Importantly, UPR

397   augmentation significantly reduced the cytolytic effect of IFNs on HBsAg accumulating

398   hepatocytes. Our data highlights an as yet unknown characteristic of the IFN signaling-UPR

399   axis that potentially presents important targets for regulating ER-stress associated cell death.

400   The HBV envelope consists of three closely related envelope proteins: small (S),

401   middle (M), and large (L), all of which have identical C-terminal ends [35]. The large

402   envelope protein (LHBs) is filamentous and tends to accumulate in the ER [36]. Lineage 107-

403   5D transgenic mice used in this study produce LHBs predominantly, and were shown to be

404   exquisitely sensitive to IFNγ [31,34,37,38]. IFNγ was also shown to induce cell death in

405   oligodendrocytes that accumulate MHC-1 heavy chains [39]. However, little is known about

406   the mechanism by which IFNγ induces cell death in ER stress-accumulating cells. In contrast,

407   the impact of type I IFNs on ER stress-accumulating cells had not been examined. Due to

408   intracellular pattern recognition receptors (PRRs) such as RIG-I, IFNα can be specifically

409   induced by virus-infected cells [3]. Less understood was whether and how IFNα specifically

410   targets virus-infected cells to exert its antiviral activity. Although many viruses are shown to

411   induce ER stress and UPR [14–19] , the interaction between IFNs, ER stress, and UPR

412   remains largely unexplored. To our knowledge, this is the first study that demonstrates the

413   direct and specific cytotoxicity of IFNα on ER stress-accumulating cells.

414   Importantly, the suppressive effect of IFN on UPR was evident not only in HBs-Tg

415   mice (Fig 4) and HBV infected chimeric mice (Fig 5), but also on chemically induced UPR

19

416   (Fig 6D and 6E). IFNα suppressed the expression of phosphorylated and unphosphorylated

417   PERK, which is a key UPR molecule presumed to suppress protein synthesis [40]. In HBs-

418   Tg mice and HBV infected chimeric mice, IFNα also suppressed the expression of spliced

419   XBP1. XBP1 is a key transcription factor that binds to unfolded protein response elements

420   (UPRE) found in several genes encoding molecular chaperones and ER-associated

421   degradation (ERAD) [41]. IFNα did not suppress XBP1 in thapsigargin-treated PMHs. Of

422   note, thapsigargin treatment did not increase LDH in the supernatant, indicating that the

423   nature of ER stress caused by HBs-Tg mice and thapsigargin is very different. Previous

424   studies have shown that hepatic deficiency of PERK [42] or XBP1 [43,44] renders

425   hepatocytes hypersusceptible to ER stress-related cell death and disease. It is currently

426   unclear whether the suppression of these molecules is the primary cause triggering the

427   cascade of cell death after IFN treatment. Knockdown of the key UPR related molecules such

428   as XBP1 and PERK individually by siRNA did not induce cell death in the absence of IFNs.

429   It is possible that the simultaneous suppression of several UPR molecules or other unknown

430   factors induced by IFNs is required to initiate the cytolytic program. Alternatively, the

431   suppression of UPR by IFN signaling may be cytolytic only when GRP78 is highly

432   upregulated. Regardless, the current study demonstrated the detrimental effect of UPR

433   suppression on HBsAg accumulating hepatocytes.

434   GRP78 was upregulated after IFN treatment selectively in HBs-Tg mice, and its

435   upregulation was clearly associated with liver injury. Because GRP78 regulates the UPR

436   through direct interaction with ER stress sensors [45], it is reasonable that the upregulation of

437   GRP78 results in reduced UPR. Paradoxically, the main function of GRP78 is thought to

438   facilitate protein folding to reduce ER stress, and its expression is also induced by UPR

439   stimulation [46]. Ample evidence suggests the critical role of GRP78 in cancer cell survival

440   and proliferation. Site-specific deletion of GRP78 in the prostate epithelium suppresses

20

441   prostate tumorigenesis [47], and knockdown of GRP78 sensitizes various cancer cells to

442   chemotoxic, anti-hormonal, DNA damaging, and anti-angiogenesis agents [48]. In stark

443   contrast to these reports, more recent studies point to a pro-apoptotic role of GRP78

444   translocated to the cell surface. Ligation of a proapoptotic protein, prostate apoptosis

445   response-4 (Par-4), to GRP78 on cell surface induces apoptosis [49] . Experiments are

446   currently underway to test whether IFNs induce GRP78 translocation to the surface of

447   HBsAg accumulating hepatocytes.

448   CHOP does not seem to play a significant role in IFNα mediated cell death associated

449   with HBsAg accumulation in our setting (Fig 7B), although it was highly induced in direct

450   correlation with ALT elevation (Fig 4E). This observation appears to contradict the widely

451   accepted notion that CHOP sensitizes cells to ER stress-mediated death. For example, cells

452   lacking CHOP are significantly protected from the lethal consequences of ER stress [50,51].

453   However, mouse embryonic fibroblasts (MEFs) derived from CHOP-knockout mice exhibit

454   only partial resistance to ER stress-driven apoptosis [50]. Furthermore, liver-specific CHOP

455   knockdown had no impact on liver damage associated with urokinase plasminogen activator

456   (uPA) accumulation in the ER [52]. Thus, it is possible that the role of CHOP depends on the

457   organs and nature of ER stress.

458   Clinically, we do not know to what extent the observed phenomenon contributes to

459   the liver disease in HBV infected patients as HBV is a poor IFN-1-inducer [53]. However,

460   liver damage is one of the most common side effects of IFNα treatment [54]. In addition,

461   accumulating evidence suggests that ALT flares during chronic HBV infection are associated

462   with increases in serum IFN-1s [23,24]. Liver injury is often severe in CHB patients

463   superinfected with hepatitis C or hepatitis D viruses [55,56] , both of which induce IFN-1s.

464   While activated NK, NKT, and T cells have been assumed responsible for the liver injury

465   associated with IFNs [23,24], the current study newly identifies UPR downregulation as a

21

466 potential mechanism of IFN-mediated hepatoxicity. One may argue that the levels of HBsAg

467 accumulation in the HBs-Tg mice cannot be attained in HBV infected patients. However,

468 some chronic hepatitis B patients, particularly those carrying HBV PreS1/PreS2 mutants [57–

469 59], exhibit ground glass hepatocytes (GGHs), a manifestation of HBsAg accumulation in the

470 ER, and tend to develop hepatic flares. Immune suppressed CHB patients occasionally

471 experience fibrosing cholestatic hepatitis, a severe form of hepatic flares, and exhibit GGHs

472 [20,21]. GGHs have been reported even in HBV-infected chimeric mice in association with

473 very high levels of intrahepatic HBV proteins and also mild hepatitis [60]. It is important to

474 point out that UPR was induced in HBV infected human hepatocytes in chimeric mice, and

475 UPR downregulation was observed in these mice after IFNα treatment, indicating that the

476 similar events observed in HBs-Tg mice could occur during bona fide HBV infection.

477　　　In conclusion, the results described herein suggest the previously unappreciated

478 mechanism by which IFNs selectively induces cell death in ER stress accumulating cells.

479 This mechanism may have evolved to selectively eliminate stressed cells due to virus

480 infections or other causes. On the other hand, the same mechanism potentially induces

481 chronic inflammation. Further studies are warranted to determine whether the IFN-UPR axis

482 contributes to the development of other ER stress-associated chronic inflammatory diseases,

483 such as alcoholic and non-alcoholic steatohepatitis, and neurodegenerative disorders.

484

Materials and Methods

485

Ethics statement

486

487 All experiments involving mice were performed in the Center for Experimental Animal

488 Science at Nagoya City University, following a protocol approved by the Institutional Animal

22

489   Care and Use Committee of the Nagoya City University Graduate School of Medical

490   Sciences (approved number: H30M_45).

491

Mouse models and treatments

493   Mouse care and experiments were performed at the Nagoya City University Center for

494   Experimental Animal Science following a protocol approved by the Institutional Animal Care

495   and Use Committee. Ten to twelve-week-old male mice were used in all the experiments.

496   HBV transgenic mice (Lineage 1.3.32) and HBs-Tg mice of the lineage 107-5D used in this

497   study were kindly provided by Dr. Francis V. Chisari. HBs-Tg mice produce filamentous

498   HBs proteins under the control of the albumin promoter, as previously described[27]. HBV-

499   Tg mice produce infectious Dane particles and HBV subviral particles as described

500   previously [28]. Human hepatocyte chimeric mice were generated by repopulating the livers

501   of severe combined immunodeficient mice transgenic for the urokinase-type plasminogen

502   activator gene (uPA$^{+/+}$/SCID$^{+/+}$ mice) with human hepatocytes, and purchased from Phoenix

503   Bio Co., Ltd, (Hiroshima Japan). Chimeric mice were infected with HBV as previously

504   described [15,] and 3-4 mice/group subcutaneously received 25ng/g of pegylated-human

505   interferon α (Peg-hIFNα-2a) (Hoffmann La Roche, Basel, Switzerland). To activate IFN-1

506   signaling, poly I:C (Sigma) (10 μg/mouse) was injected intravenously. To block type-1 IFN

507   signaling, 250μg anti-IFN$\alpha\beta$R1 antibody (clone MAR1-5A3) or isotype control (IgG1)

508   (BioXcell, Lebanon, NH, USA) were injected intraperitoneally. To test the effect of IFNα, 5

509   million units per kg recombinant mouse IFNα (Miltenyi Biotec) were injected intravenously.

510   To upregulate UPR, 0.1mg/kg tunicamycin was injected intraperitoneally (Merck).

511

Serum ALT and HBsAg analyses.

513   Serum ALT was measured using the Dri-Chem 3500 analyzer according to the

514   manufacturer's instructions (Fuji, Tokyo, Japan). Serum HBsAg and intrahepatic HBsAg

515   were measured by a chemiluminescent enzyme immunoassay (CLEIA) using a

516   LumipulseG1200 analyzer (Fujirebio, Tokyo, Japan), as previously described [33].

517

518   **Detection of DNA fragmentation by TUNEL method.**

519   Mouse livers were perfused with PBS, harvested in Zn-formalin and transferred into 70%

520   ethanol 24 hours later. Tissue was then processed, embedded in paraffin and stained as

521   previously described [61]. In situ apoptosis detection was carried out by using a Terminal

522   deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) apoptosis kit, according to

523   the manufacturer's instructions (ab206386; Abcam Biotechnology, Cambridge, UK). The

524   samples were counterstained with Mayer's hematoxylin for the morphological evaluation and

525   characterization of normal and apoptotic cells.

526

527   **Adoptive HBsAg-specific T cell transfer and In vivo HBsAg suppression.**

528   HBsAg-specific CD8+ T cells, derived from TCR transgenic mice (Env-28 specific, Lineage:

529   6C2.16) [62], were simulated in-vitro for one-week as previously described [31]. These cells

530   were intravenously injected into groups of HBs-Tg mice at a dose of 5 x10^6 cells/mouse.

531   Groups of HBs-Tg mice were intravenously injected with a single dose (5mg/kg) cocktail of

532   HBV specific siRNA (si75, si251, si1803)[29,30,63] or Control siRNA complexed with a

533   pH-sensitive multifunctional envelope-type nanodevice (MEND) [29].

534

535   **Immunoblots**

536   Whole-cell extracts were obtained from liver tissue or cell pellets lysed in buffer (0.1%,

537   sodium dodecyl sulfate, 0.1% sodium deoxycholate, 1% IGEPAL) supplemented with

538   Protease and Phosphatase Inhibitor cocktails (Roche). Protein extracts were separated by

539   SDS-polyacrylamide gel electrophoresis then transferred onto polyvinylidene difluoride

540   (PVDF) membranes (Millipore, Temecula, CA). Primary antibodies and secondary antibodies

541   were used according to manufacturers' instructions. Primary antibodies used are GRP78

542   (#3177), CHOP (#2895), XBP1s (#12782), phosphor-PERK (#3179) PERK (#3192), ISG15

543   (#2743) IRE1α (#3294), Stat-1 (#9172), phospho-Stat-1 (#9167) (all from Cell Signaling

544   Technologies), GAPDH (abcam#8245), HBsAg (#5124A, Tokumen, Japan) phospho-IRE1α

545   (Novus #NB100-2323) Detection was performed using the Immobilon Western

546   Chemiluminescent HRP Substrate (Millipore) and image capture was done using the A1680

547   imaging system (GE Healthcare).

548

549   **RNA extraction and gene expression analyses**

550   RNA was isolated from snap-frozen liver tissue obtained at selected time points using Isogen

551   (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. For Microarray

552   analyses, 2 biological replicates for each selected time point were analyzed. Briefly, from

553   100ng total RNA, complementary RNA (cRNA) was prepared using the Low Input Quick-

554   Amp Labeling Kit, one color Cy3 protocol (Agilent Technologies, Santa Clara, CA, USA).

555   Purified labeled-cRNA and controls (Agilent One Color Spike-In Kit) were hybridized to

556   Agilent SurePrint G3 Mouse Gene Expression v2.0 Microarray Chips. Detection, data

557   extraction, and pre-analysis were performed using a G2505C Agilent microarray scanner,

558   Feature Extraction v10.10.1.1, and GeneSpring GX v14.8.0 software. Genes showing

559   differential kinetics between IFN treated or non-treated HBsAg and WT samples over the

560   period leading up to the onset of liver injury in HBs-Tg mice, i.e., 0, 2, 4, and 8 hour time

561   points, were identified using the time-course specific R program maSigPro [32]. Briefly,

562   maSigPro assesses the significance of the global model (i.e., if there are significant

563   differences with respect to time or treatment) and of each variable (i.e., which specific time

564   or treatment change is present) by fitting a regression model, considering time as a

565   continuous variable and creating specific variables for each treatment, thereby adjusting a

566   temporal profile for each time course. Genes significantly changed were selected and divided

567   into clusters of similar profiles for visualization of results. Microarray data have been

568   deposited into the NCBI Gene Expression Omnibus Repository (Accession # GSE 138916)

569   For microarray data validation and specific target gene expression analysis, quantitative real-

570   time polymerase chain reaction (qRT-PCR) was performed using the StepOnePlus Real-Time

571   PCR System (Applied Biosystems, Foster City, CA). From 2μg total RNA, complementary

572   DNA (cDNA) was synthesized using the High Capacity RNA-to-cDNA Kit (Applied

573   Biosystems). TaqMan Gene Expression Assay primer-probe sets used include; GRP78

574   (Mm00517690_g1, Hs00607129_gH), CHOP (Mm01135937_g1, Hs03834620_s1), XBP1s

575   (Custom), PERK (Mm00438700_m1, Hs00984006_m1), GAPDH (Mm99999915_g1,

576   Hs02758991_g1), IRE1α (Mm00470233_m1), ISG15 (Mm01705338_s1, Hs01921425_s1),

577   IRF1(Hs00971960_m1) (all from Applied Biosystems). All qPCR data were normalized to

578   GAPDH.

579

580   **Isolation, culture, and treatment of primary mouse hepatocytes**.

581   Primary mouse hepatocytes were isolated from both HBs-Tg and their wildtype littermates

582   (controls) using a two-step protocol as previously described [62] with some modification.

583   Briefly, the liver was perfused with Liver Perfusion Medium (Gibco #17701) for 4 minutes at

26

584   a flow rate of 5ml/min followed by 0.8mg/ml Type 1 Collagenase (Worthington, UK) in

585   Dulbecco Minimum Essential Medium (Gibco # 11965-092) for 8-12 minutes at 5ml/min.

586   PMHs were cultured on Collagen 1 Biocoat 6-well plates (BD Biosciences) at a seeding

587   density of $1x10^5$cells/cm^2 in Hepatocyte Growth Medium with 2% DMSO. Thapsigargin

588   (5μM, Fujifilm-Wako, Osaka, Japan) was added to IFNα-treated WT-PMHs that were then

589   harvested after 6 and 12 hours. Recombinant mouse IFNα was added at 0.1MU/ml.

590   Recombinant mouse IFNγ was added at 0.01MU/ml per well.

591

592   **LDH activity assay.**

593   Cytotoxicity was measured by the release of lactate dehydrogenase (LDH) into the culture

594   media 72 hours after the addition of IFNα (final concentration, 0.1MU/ml). Collected

595   supernatants were assayed using the Dri-Chem analyzer according to the manufacturer's

596   instructions (Fuji, Tokyo, Japan). Data are presented as percentage specific cell lysis,

597   calculated as the ratio of the experimental LDH release (minus spontaneous release LDH) to

598   the maximum LDH release using 1% Triton-X (minus spontaneous release LDH) for each

599   plate.

600   % specific lysis = LDH [(experimental -spontaneous)/maximum lysis – spontaneous] x100

601

602   **Knockdown of UPR-related target genes**.

603   To suppress UPR related molecules in vivo and in vitro, the Invivofectamine 3.0 and

604   Lipofectamine RNAiMAx transfection reagents were used respectively, together with siRNA

605   for the following targets; Control (#1), GRP78 (s607083, s67085), XBP1 (s76114), CHOP

606   (s64888), all according to manufacturer's instructions (all from Thermofisher Scientific).

27

607

608 **Statistics**.

609 Student's t-test and One-Way ANOVA were performed accordingly. Microarray data

610 bioinformatic analyses were performed as previously described [32]. Data are depicted as the

611 mean ± SD, and P values < 0.05 were considered significant.

612

613

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619

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Competing interests

646   The authors have declared that no competing interests exist.

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Supporting information

S1 Fig

Reduction of intrahepatic HBsAg. (A) Experimental design. In vivo suppression of HBsAg using siRNA. HBs-Tg mice were intravenously injected with mixed siRNA targeting HBV (5mg/kg) then sacrificed at the specified time points. (B) The graph shows >10 fold reduction in HBV mRNA levels by siHBV treatment. (C) HBsAg levels in HBs-Tg mice treated with siHBVcompared with control mice.

S2 Fig

(A) Baseline expression of UPR markers in non-treated 107-5D HBs-Tg mice. The Immunoblots show the expression of UPR molecules like CHOP, XBP1 and GRP78 in non-treated HBs-Tg mice compared with normal WT mice. (B) Serum ALT levels in non-treated HBs-Tg mice compared with normal WT mice.

S3 Fig

Characterization of the baseline extracellular and intracellular HBsAg levels in non-treated HBV infected chimeric, HBV-Tg and HBs-Tg mice. (A) A graph showing the serum HBsAg levels between chimeric mice, HBV-Tg and HBs-Tg mice. (B) A graph showing the intracellular HBsAg levels per μg of liver protein between chimeric mice, HBV-Tg and HBs-Tg mice.

S4 Fig

IFNγ is directly cytotoxic to hepatocytes accumulating HBsAg. (A) Timeline showing how primary mouse hepatocytes (PMHs) from both HBs-Tg and WT mice were isolated, plated, and treated with either IFNγ, while monitoring cytotoxicity at the specified time points. (B) The graph shows significant LDH increase in HBs-Tg derived PMHs 72 hours after IFNγ addition. (C) The immunoblots show the effect of IFN-1 signaling on UPR-related proteins at specified time points after IFNγ treatment.
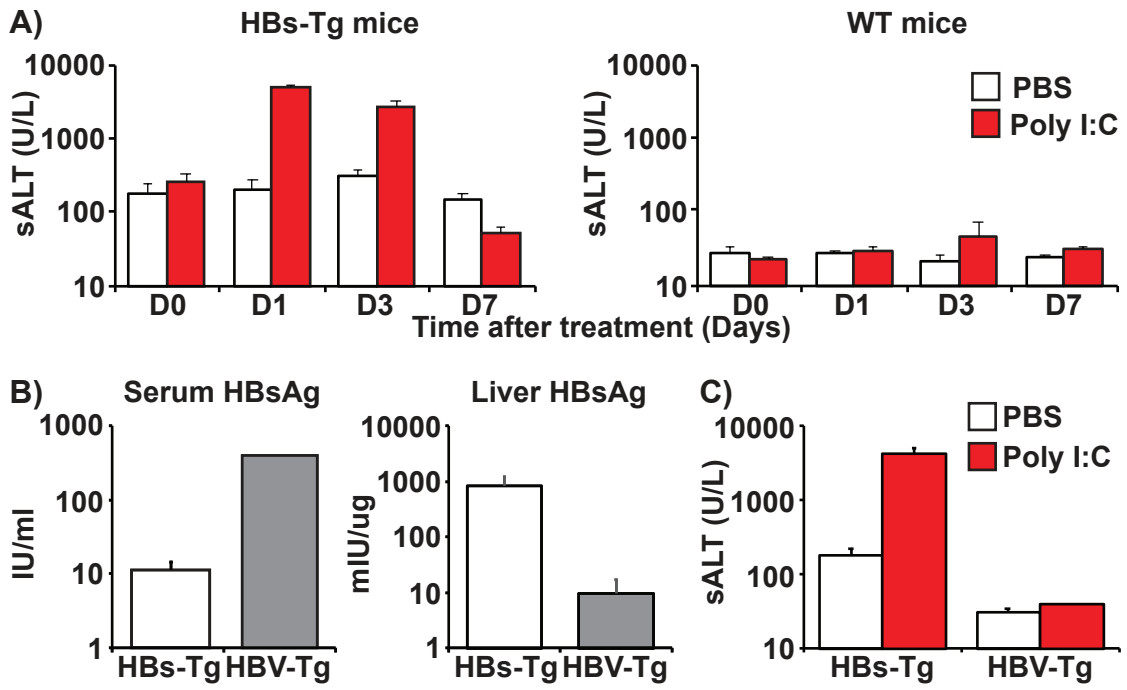
Fig 1

A) HBs-Tg mice / WT mice

B) Serum HBsAg / Liver HBsAg

C)

Fig 2

A)

sALT (U/L) vs Time after treatment (Days): D0, D1, D3, D7

Legend:
- Ig control+Poly I:C
- anti-IFNαβR1+Poly I:C

B)

HBs-Tg mice | WT mice

sALT (U/L) vs Time after treatment (Days): D0, D1, D3, D7

Legend:
- Vehicle
- IFNα

C)

H&E (24 hrs after IFNα)

HBs-Tg | WT

sALT 3520 U/L   sALT 26 U/L

D)

TUNEL (16 hrs after IFNα)

HBs-Tg | WT

sALT 5020 U/L   sALT 31 U/L

Fig 3

A)

Day -7 — HBs-specific CD8+ T cells — HBs-Tg

Day 0 — siHBV / siControl

Day 7 — IFNα i.v.

Day 8 — sALT
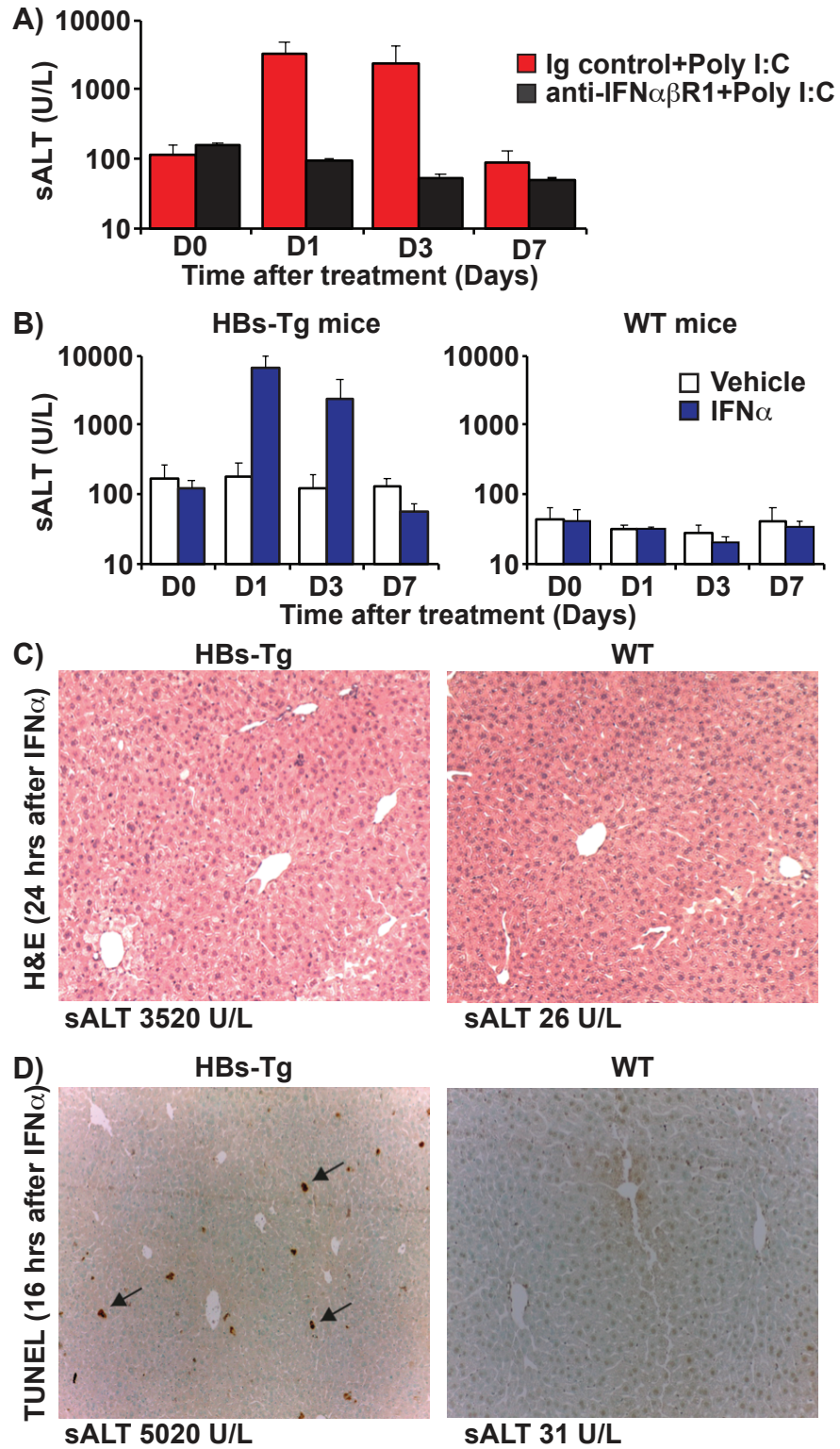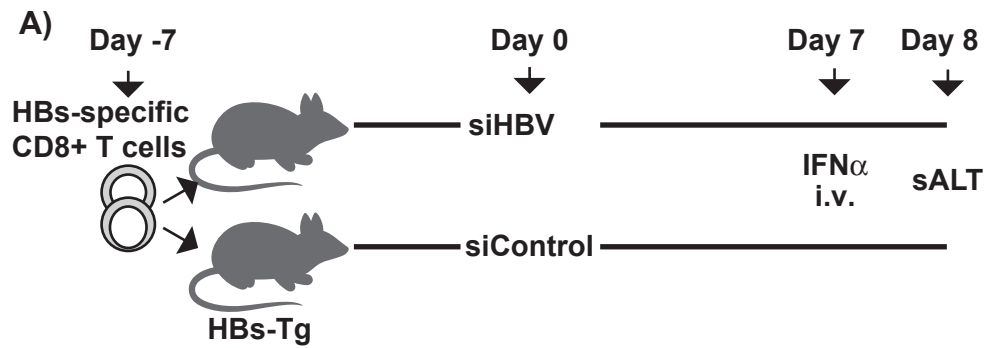
B) Intrahepatic HBsAg on Day 7

siControl | siHBV

LHBs
mHBs
sHBs
Gapdh

C) Serum ALT

p=0.027

siControl
siHBV

sALT (U/L)

10000 / 1000 / 100 / 10

Pre-IFN (Day 7) | Post IFN (Day 8)

Fig 4

A) Liver injury

B) Cluster 1:(1018genes) Cluster 2:(182genes) Cluster 3: (692genes) Cluster 4:(1032genes) Cluster 5: (404genes) Cluster 6 (330genes)

C) Cluster

Spliceosome
Endocytosis
MAPK signaling
Basal transcription factors
Pathways in cancer
Proteoglycans in cancer
NF-Kb signaling
DNA replication
Cell cycle
Metabolic pathways
ABC transporters
Bile secretion
Alzheimer's disease
Oxidative phosphorylation
Parkinson's disease
NAFLD
Alcoholism
ER protein processing
Phagosome
Olfactory transduction
Neuroactive l-r interaction
Cell adhesion molecules
Calcium signaling
Herpes simplex infection
Allograft rejection
Influenza A
Autoimmune thyroid disease
TLR signaling
Chemokine signaling
Fc-y R-med phagocytosis
TNF signaling
Hepatitis B
Hepatitis C

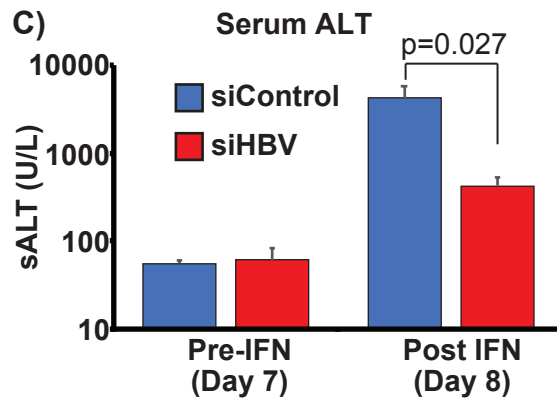**D)** ER STRESS, UPR
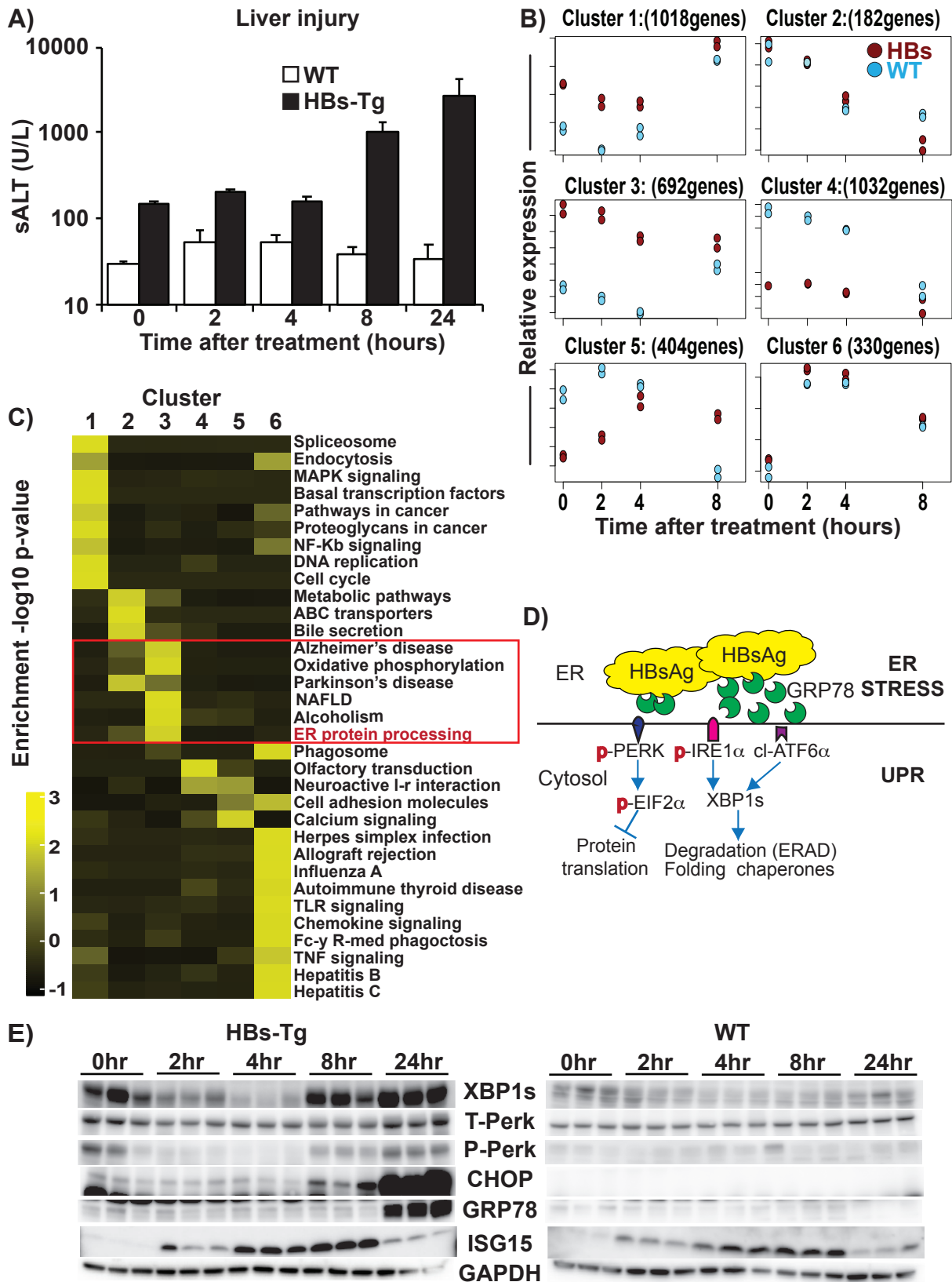
E) HBs-Tg / WT: XBP1s, T-Perk, P-Perk, CHOP, GRP78, ISG15, GAPDH

Fig 5

A) HBV infected human chimeric mice

B)
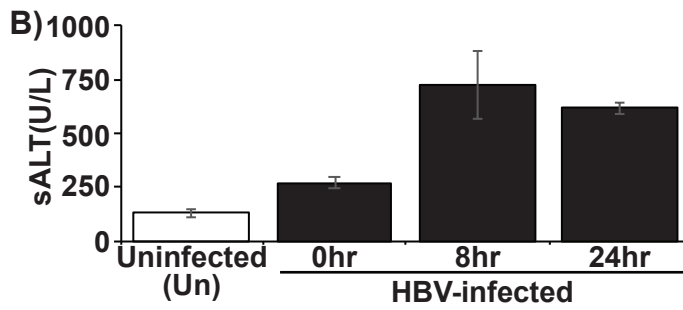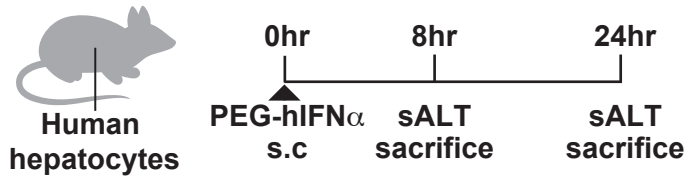

C)

D)

Fig 6

A)

HBs-Tg or WT mice → Primary Mouse Hepatocytes (PMH)

Harvest

-24hr ▲Plating 0hr ▲IFNα 8hr 24hr 72hr

B)

Cytotoxicity

% Specific lysis (y-axis: 0, 25, 50, 75, 100)

□ Medium   ■ IFNα

**

0 8 24 72 — HBs-Tg   0 8 24 72 — WT

C)

UPR

HBs-Tg   WT

0 8 24 72   0 8 24 72

GRP78
XBP1
T-Perk
P-Perk
CHOP
ISG15
GAPDH

D)

Harvest

-48hr ▲Plating WT PMH   -24hr ▲IFNα   0hr ▲Thapsigargin (5µM) 6hr 12hr

E)

0hr   6hr   12hr

IFNα-   IFNα-   IFNα+   IFNα-   IFNα+

XBP1s
T-Perk
P-Perk
CHOP
GRP78
ISG15
GAPDH

Fig 7

A)

HBs-Tg → Primary Mouse Hepatocytes

-48hr | -24hr | 0hr | 72hr
Plating | siRNA | IFNα | Harvest

B) Cytotoxicity

p=0.014

% Specific lysis

siCTRL, siCHOP, siGRP78, siXBP1

C)

| | siCTRL | siCHOP | siGRP78 | siXBP1 | siCTRL | siCHOP | siGRP78 | siXBP1 |
+IFNα

ISG15
GRP78
XBP1s
T-Perk
P-Perk
CHOP
GAPDH

D) Cytotoxicity

p=0.024 p=0.023

% Specific lysis

siCTRL, siGRP78, siGRP78/siXBP1

E)

+IFNα

siCTRL: + , +
siGRP78: + , + , + , +
siXBP1: + , +

ISG15
GRP78
XBP1s
TPerk
P-Perk
CHOP
GAPDH

Fig 8

A) GRP78 suppression

Day -4   Day 0   Day 1

siGRP78   IFNα   sALT
or siCTRL

B)

C) Low dose Tunicamycin

-4hr   0hr   24hr

TUN   IFNα or αGal   sALT
or Vehicle

D) IFNα

E) αGal

F)

S1 Fig

A) HBs mRNA suppression

Day 0   Day 4

siHBV or   HBs Protein
siControl   and HBV mRNA

B) HBs Protein

C) HBV mRNA

S2 Fig

A)

B)

S3 Fig

A)

Serum HBsAg

B)

Liver HBsAg

S4 Fig

A)

B)

C)